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13. ABSTRACT *(Maximum 200 words)*

The research supported by this grant is intended to evaluate Rak mRNA expression in both tumor tissue and breast cancer cells and to determine whether inhibiting RAK is a feasible approach to breast cancer therapy. To accomplish this goal two different assays of gene expression have been devised. The first method is a traditional competitive RT-PCR system in which a homologous competitor RNA is used as a standard. The second method involves the use of an electrochemical biosensor being developed in our lab. The biosensor is designed to detect the abstraction of electrons from guanine bases in surface immobilized target RNA's or RT-PCR products. This report describes the development of a rapid electrochemical method that has detected Rak RT-PCR products. Preliminary characterization of this system has determined the sensitivity limit of this system to be 60 amol/mm^2 of electrode. In addition, results from competitive RT-PCR experiments on Rak mRNA have revealed that RAK is expressed in BT-474 cells at a level of roughly 100 zmol/μg total RNA.

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Paul M. Armistead 7/27/99

PI - Signature Date

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Introduction:

The objectives of the research covered through this grant are twofold. The first objective involves monitoring of Rak mRNA expression in cultured cell lines and tumor tissue through two different bioassays: a quantitative RT-PCR system, and a rapid electrochemical method being developed in our lab. The electrochemical detection scheme under investigation is based upon quantification of the catalytic transfer of electrons from guanine bases in a surface immobilized target mRNA or RT-PCR product to an electrode.[1] This report describes the development of this electrochemical biosensor and its use in quantifying Rak RT-PCR products. The report also includes the development of a competitive RT-PCR system that has enabled us to determine the absolute expression of Rak in BT-474 cells. With the completion of this first objective, the second objective, the determination of Rak and Cdc2 mRNA expression in cells microinjected with a Rak peptide can now be undertaken.

Body:

The electrochemical system used in the detection of mRNA is based upon quantification of electrons transferred from the guanines in a specific gene product to an indium tin oxide (ITO) electrode upon which the gene product has been immobilized. Because electron transfer from guanine to all electrode materials is slow, a small soluble oxidation catalyst, $Ru(bpy)_3^{2+}$ (bpy = 2,2' bipyridine), is added to the system to greatly increase the rate of electron transfer and the amount of current detected in the system.[1,2] The catalyst is oxidized at the electrode surface (eq 1) and is then reduced by guanines present in the system which regenerates the catalyst (eq 2).

$$Ru(bpy)_3^{2+} \rightarrow Ru(bpy)_3^{3+} + e^- \quad (1)$$

$$Ru(bpy)_3^{3+} + \text{guanine} \rightarrow Ru(bpy)_3^{2+} + \text{guanine}^+ \quad (2)$$

Because the number of electrons transferred is proportional to the guanine content of a specific nucleic acid, electrochemical quantification of a certain gene product should be feasible provided the sequence of the gene of interest is known.

This system was first characterized with both the nucleic acid and the $Ru(bpy)_3^{2+}$ in solution.[1] However, the amount of nucleic acid required for this method was prohibitive, and it was not obvious how detection by hybridization could be achieved with a purely solution based system. Switching to a system that involved immobilizing the nucleic acid on the surface of the electrode has solved both problems. Because electrochemical techniques detect chemical reactions at a surface, immobilization of DNA onto the electrode greatly reduces the amount of substrate required without affecting the sensitivity of the system. Also, the immobilization of a probe strand with inosine (which is electrochemically silent) substituted for guanosine can create a hybridization biosensor which should hybridize a complementary strand of interest. In the past year we have developed a our most promising method for rapidly immobilizing DNA onto ITO electrodes in a way that is highly reproducible. The immobilization reaction involves adding purified PCR products in 100 mM sodium acetate buffer to 9 volumes of N,N'-dimethyl formamide (DMF). The mixture is pipetted onto an ITO electrode and is adsorbed in under 15 minutes. The reproducibility of this system has proven to be very good, and detection of trace amounts of DNA has been achieved.

Progress has also been made in the determination of the absolute level of Rak expression in BT-474 cells. A competitive RT-PCR strategy has been developed in which an exogenous RNA competitor is added to the total RNA extracted from the BT-474 cells. The competitor is synthesized by performing RT-PCR of Rak mRNA in total RNA. The PCR primers are designed so that the sense primer contains a sequence that will force a loop-out region to form in the complementary strand upon hybridization. This primer also

contains the T7 RNA polymerase promotor sequence at its 5' end. After the round of RT-PCR is complete, a DNA is produced that is shorter than the normal Rak product because of the loop-out. Because this product also contains the T7 promotor sequence, *in-vitro* transcription can be performed to produce a shortened Rak competitor with the same PCR primer sites as native Rak.

Results. Rak RT-PCR products were adsorbed onto ITO electrodes from a 9:1 DMF:acetate mixture. The 250 b.p. Rak amplicon was synthesized as described in the experimental section, and varying concentrations of the product were applied to different ITO electrodes. After thorough rinsing of the DNA modified electrodes, they were exposed to a dilute solution of $Ru(bpy)_3^{2+}$ and analyzed by cyclic voltammetry performed at a high scan rate (Figure 1). The peak current observed was proportional to the concentration of the DNA mixture applied to the electrode, with the current generated from $Ru(bpy)_3^{2+}$ alone being very small.

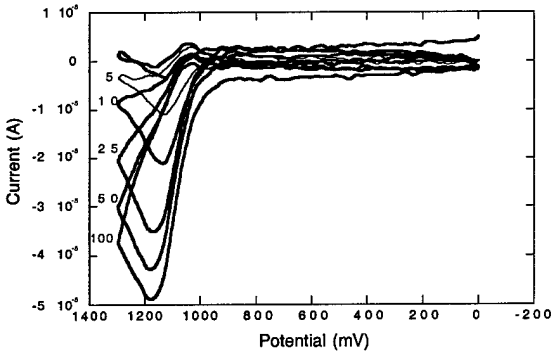

Figure 1. Representative cyclic voltammograms of $Ru(bpy)_3^{2+}$ on DNA-modified electrodes. The voltammograms show ITO's treated with DMF/acetate solutions containing 100, 50, 25, 10, 5, and 0 μM Rak RT-PCR product (nucleotide concentration).

The basic characteristics of this electrochemical system have been determined through a combination of experiments involving RT-PCR products of the HER-2/neu gene. The electrochemical current detected is proportional to the amount of guanines immobilized on the electrode surface. The experiments that established this relation were based upon 3 different sized RT-PCR products (435 b.p., 1020 b.p. 1497 b.p.) synthesized from HER-2 mRNA. Electrochemical measurements of DNA-modified electrodes treated with different concentrations of these PCR products were correlated with data from DNA modified electrodes treated with identical PCR products that were body labeled with P^{32}-dCTP (Figure 2). The correlation shows that peak current from cyclic voltammetry depends linearly upon the number of nucleotides (i.e. guanines), immobilized on the surface. These data also establish a sensitivity limit of this system of roughly 60 amol/mm^2 of electrode area for long (≈ 1500 b.p.) PCR products. With this level of sensitivity it should be possible to detect highly expressed mRNA's in cell samples (e.g. actin) at these macroscopic electrodes without amplification, but the detection of rare mRNA's, such as Rak or many other signaling molecules will probably still require amplification through RT-PCR.

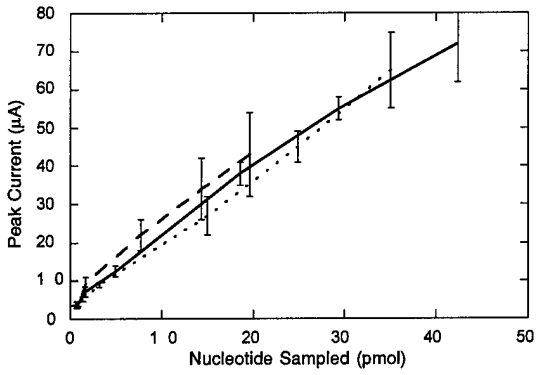

Figure 2. Dependence of the peak current from cyclic voltammograms of $Ru(bpy)_3^{2+}$ on electrodes treated with either 1497 b.p. (line), 1020 b.p. (dash) or 435 b.p. (dot). The three lines are identical, within the error of the experiment, indicating that peak current reflects the total amount of guanine attached to the surface regardless of the size of the DNA attached.

Competitive RT-PCR has been employed to determine the absolute level of Rak mRNA expression in BT-474 cells. A 250 b.p. competitor DNA template was produced that contained a T7 RNA polymerase promotor and a deletion that would be used to discriminate the competitor from the native RNA following RT-PCR. *In-vitro* transcription of the competitor yielded an RNA that was 232 bases long (64 bases shorter than the native RNA); however, both the competitor and the native Rak mRNA contained the same primer binding sites. As a result, PCR following reverse transcription could proceed in one tube with the use of only two primers for both sequences.

In the competitive RT-PCR experiments, increasing amounts of competitor RNA (ranging from 1 zmol to 10 amol) were added to reverse transcription reactions that contained 1 μg of total BT-474 RNA. Because the two templates compete for enzyme, primers and nucleotides in both the reverse transcription and PCR steps, the amount of both products produced after PCR accurately reflects the initial starting amounts.[3] Because the initial amount of competitor RNA added to the reaction is known, the amount of target (i.e. Rak) mRNA can be calculated. Early experiments with this system revealed that Rak is expressed at a level of roughly 100 zmol/μg RNA. With optimization, a more accurate value can be determined.

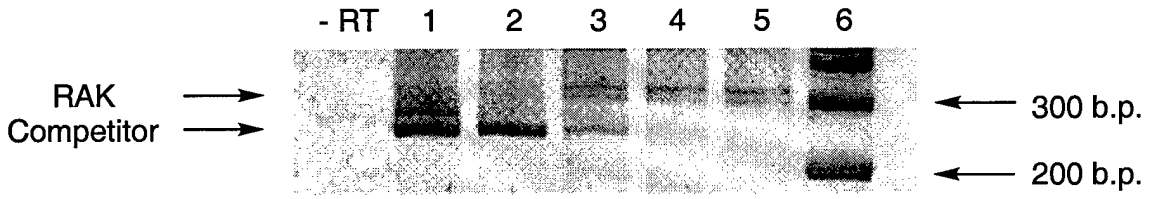

Figure 3. Competitive RT-PCR of Rak mRNA. Lanes 1-5 have competitor RNA at 10 amol, 1 amol, 100 zmol, 10 zmol, and 1 zmol respectively. Rak mRNA shows a band at 296 b.p. Lane 6 is a 100 b.p. ladder, and - RT is the same reaction as in lane 1 except Reverse Transcriptase was not added in the reverse transcription.

Experimental. BT-474 cells were provided by our collaborators in the lab of Dr. Bill Cance at the UNC Lineberger Comprehensive Cancer Center. RNA was extracted and

purified by means of an RNeasy RNA purification kit from Qiagen. Reverse transcriptions performed on total RNA were carried out by mixing 2 μL of total RNA (0.8 μg/μL), 2 μL Random hexamers (Promega), 4 μL dNTP's (10 mM, Amersham) and 6 μL RNase free water. The reverse transcription mix was heated to 80°C for 3 min. to denature the RNA and then immediately placed on ice. To the mix was added 1 μL RNasin (Promega), 1 μL M-MLV Reverse Transcriptase (Life Technologies, 200 U/μL), and 4 μL 5X Reverse Transcription Buffer (Life Technologies). The reactants were mixed and incubated at 37°C for 1 hr.

RT-PCR of the Rak mRNA was used to create sufficient material for electrochemical detection. A 50 μL PCR mix was assembled from 5 μL of a reverse transcription reaction, 2.5 μL of the forward primer - GGC CTA TCT GGA GTC TCG GAA (5 μM), 2.5 μL of the reverse primer - GGG CAC CTG TCA TAC CAC TGT (5μM), 2.5 μL $MgCl_2$ (50 mM), 2.5 μL dNTP's (10 mM), 5 μL 10X PCR buffer, 30 μL water, and 1 U Taq polymerase (Life Technologies, 5 U/μL). The 296 b.p. product was produced as follows: 1 cycle at 95°C for 5 min., 35 cycles of 94°C for 20 sec, 62°C for 30 sec, 72°C for 40 sec., and 1 cycle of 72°C for 5 min. One band of the appropriate weight was observed by agarose gel electrophoresis. Primers and unincorporated nucleotides were removed from the RT-PCR products via a Concert PCR Purification kit (Life Technologies). The purified products were used as templates for further amplifications as more product was needed.

Tin doped indium oxide coated electrodes were cleaned according to the following procedure. ITO electrodes were sonicated in an alconox solution (8 g alconox per liter water) for 15 min. The electrodes were rinsed and sonicated in isopropanol for 15 min. The electrodes were then sonicated in water for 15 min. This final step was repeated, and the electrodes were allowed to dry. Purified PCR products were immobilized onto the ITO surface by mixing 5 μL of the PCR product in 100 mM sodium acetate buffer, pH = 6.8, with 45 μL dimethyl formamide (DMF). The 50 μL mixture was pipetted onto the ITO, completely covering the electrode. The electrode was placed in a constant humidity chamber for one hour because preliminary experiments with the system showed that a maximum amount of calf thymus DNA was immobilized on the ITO by this method within 15 minutes. The electrodes were then rinsed by immersing them in solutions that were agitated on a rotary mixer (Thermolyne). The electrodes under went two water washes each one for 3 min., one wash in 1 M sodium chloride for 5 min., one wash in 100 mM sodium phosphate (pH = 7.0) for 5 min., and three final washes in water each for 3 min. The electrodes were then allowed to dry. The amount of DNA attached to the ITO surface was controlled by changing the concentration of the PCR product that was applied to the ITO.

Electrochemical detection of the surface immobilized PCR products was performed on a BAS 100 B/W potentiostat connected to a 200 MHz pentium computer. All experiments were performed in a one compartment electrochemical cell. The electrode's geometric surface area was calculated to be 12.6 mm^2. A mini Ag/AgCl (Cypress Systems) electrode was used as the reference, and a platinum wire was used as the counter electrode. Cyclic voltammetry was performed from 0 to 1,300 mV vs. Ag/AgCl at a scan rate of 10 V/s. For each batch of electrodes cleaned, one electrode was used to perform a background scan. This electrode was not treated with DNA; however, cyclic voltammetry was performed on it at the above parameters in 50 mM sodium phosphate, pH = 7.0. Cyclic voltammetry was performed on the DNA modified electrodes according to the parameters above in a solution of 10 μM $Ru(bpy)_3^{2+}$ in 50 mM sodium phosphate, pH = 7.0. The cyclic voltammograms of the DNA modified electrodes were background subtracted, and the peak currents were measured. Cyclic voltammograms that produced negative signals were not used in further analysis. Poor background subtraction only became a significant source of error at final DNA concentrations of 5 μM (concentration of nucleotides in the buffer/DMF mixture) and below.

Radiolabelled PCR products were synthesized according to the method of Mertz and Rashtchian.[4] The doped PCR products were attached to the ITO surface in an identical manner to that of the non-labeled samples and were washed identically also. After the electrodes dried, they were wrapped in Saran Wrap (Dow) and placed on a phosphorimager screen (Molecular Dynamics). Aliquots of 1 μL of certain concentrations from the labeled PCR products were applied to filter paper, dried, and also wrapped in Saran Wrap. They were placed on the same phosphorimager screen and used as standards. The phosphorimager screen was exposed for approximately 12 hours and scanned on a Molecular Dynamics Storm 840 phosphorimager.

Synthesis of the Rak competitor template DNA was performed using the above procedure for reverse transcription followed by the PCR described in the synthesis of the Rak PCR product used in the electrochemical measurements. The forward primer for this PCR was TAA TAC GAC TCA CTA TAG GGC CTA TCT GGA GTC TCG GAA GCA GAT TTT GGA CTT GCC AGA, and the reverse primer was GGG CAC CTG TCA TAC CAC TGT. Synthesis of the appropriate product was confirmed by detection of a 250 b.p. product on a 2% agarose gel. *In-vitro* transcription was performed on the competitor template by mixing 4 μL template DNA (840 nM), 2 μL tris (400 mM, pH-7.5), 2 μL $MgCl_2$ (100 mM), 2 μL DTT (100 mM), 2 μL NTP's (20 mM each NTP), 2 μL bovine serum albumin (New England Biolabs, 500 μg/ml), 4 μL DEPC water, 1 μL RNasin (Promega) and 1 μL T7 RNA polymerase, which was a gift from the lab of Kevin Weeks. The reaction was incubated at 37°C for 7 hr., after which 1 μL of RNase free DNase 1 (Ambion, 2U/μL) was added to the reaction. The template DNA was digested for 30 min., and the reaction mixture was diluted by adding 100 μL DEPC water, 15 μL 5 M ammonium acetate, and 15 μL 100 mM EDTA. The reaction was extracted from 150 μL acid phenol/chloroform/isoamyl alcohol. The organic layer was back extracted with 50 μL DEPC water. The 200 μL total aqueous layer was divided into two 100 μL samples, and each sample was precipitated overnight by adding 300 μL of ice-cold isopropanol and storing the samples at -20° C. The samples were centrifuged at 14,000 r.p.m. for 15 min., and the supernatant removed. The pellets were each resuspended in 10 μL of TE buffer pH=7.5. The samples were gel purified on a denaturing 4% polyacrylamide gel, and visualized by UV shadowing. The gel fragments were cut with a clean razor blade and immersed into 300 μL of 0.5 M ammonium acetate, 1 mM EDTA, and 0.2% SDS. The fragments were kept in the elution buffer overnight at 4° C. The eluted RNA was ethanol precipitated and resuspended in 20 μL TE buffer pH=7.5. The total yield from this procedure was 25 μL of 1μM (25 pmol ≈ 2 μg) RNA that was 232 bases in length.

Competitive RT-PCR was performed with 5 different quantities of competitor. Six reverse transcription reactions were assembled. For each reaction 6 μL of water, 4 μL dNTP's (Amersham, 2.5 mM each), 2 μL reverse primer - GGG CAC CTG TCA TAC CAC TGT (5 μM), 2 μL BT-474 RNA (0.5 μg/μL), and 1 μL competitor RNA were mixed. The competitor RNA concentrations were 10 pM, 1 pM, 100 fM, 10 fM, and 1 fM resulting in competitor RNA amounts ranging from 10 amol to 1 zmol. Two reverse transcription reactions were treated with 1 μL of the 10 pM competitor solution. One of these reactions was used as the - Reverse Transcriptase control. After the reactions were mixed, they were heated to 80°C for 3 min., and placed on ice. To each reaction was added 4 μL 5X reverse transcription buffer (Life Technologies), 1 μL RNasin (Promega), and 1 μL M-MLV Reverse Transcriptase (Life Technologies. For the - Reverse Transcriptase control 1 μL of water was substituted for the enzyme. The reactions were incubated at 37°C for 1 hr. followed by heating at 90° for 10 min. to denature the enzyme. PCR was performed on the reverse transcription reactions according to the same parameters as those used to synthesize the competitor template. The reactions were analyzed by native PAGE on a 5% polyacrylamide gel. The gel was stained with sybr-green and analyzed on a Storm 840 phosphorimager.

Research Accomplishments

- New method for the attachment of DNA onto ITO electrodes. The method is rapid and involves no coupling reagents.
- Electrochemical detection of Rak RT-PCR products.
- Electrochemical detection of RT-PCR prodcuts at a level as low 60 amol/mm^2 of electrode.
- Determination of Rak mRNA to be expressed at roughly 100 zmol/μg total BT-474 RNA.

Reportable Outcomes

- Results in this report will be submitted as a paper in Analytical Chemistry during the next granting period and should be in print before the next annual report.
- Characterization of this electrochemcial system was presented as a poster at the National M.D./Ph.D. Conference in Aspen CO July 17, 1999.
- These data and data from the next granting period will be presented at the DoD meeting in Atlanta in June, 2000.

Conclusions

The development of a rapid method for the immobilization of DNA onto ITO electrodes has greatly facilitated the development of an electrochemical bioassay for the quantification of Rak and other mRNA's. The determination of the system's sensitivity limit of 60 amol/mm^2 of electrode indicates that Rak mRNA will have to be amplified for electrochemical detection.

A preliminary electrochemical assay can already be envisioned for the quantification of Rak mRNA after RT-PCR amplification. An assay that could measure the *relative* changes in Rak mRNA expression could be implemented by performing RT-PCR on total RNA extracted from cells treated in various ways. The PCR products could be applied to the electrode in the manner described above, and the peak current determined by cyclic voltammetry would be proportional to the amount of Rak PCR product present. The electrochemical data could be compared to traditional gel based techniques. The data from a system like this would be sufficient to answer many questions of biological importance including the questions addressed in the statement of work. The major technical problem of this system would be the difficulty in ensuring that all reverse transcriptions and PCR steps were performed under exactly the same conditions. One way to control for this error is to perform a parallel standard RT-PCR with a known amount of target RNA. The electrochemical signal from this standard could serve as a baseline for the other samples.

To determine the absolute expression level of Rak mRNA in BT-474 cells competitive RT-PCR followed by gel electrophoresis has been used. Preliminary experiments have shown that Rak mRNA is expressed in BT-474 cells at a level of roughly 100 zmol/μg total RNA. Refinement of this technique by optimizing the reverse transcription and PCR parameters and by narrowing the range of the amounts of competitor applied should yield a more accurate result soon.

With the development of the Rak competitive RT-PCR assay and the electrochemical system, Task 1b in the statement of work has been completed. We recommend that Task 1a not be pursued because it will not aid in the improvement of these assays. By using the assays described above, we plan to begin work on determining Rak and Cdc2 expression in breast cancer cells cells that are microinjected with Rak peptide 86-97 as mentioned in Task 2b. The recipient cells' growth will also be analyzed in accordance to Task 2a.

[1] Johnston, D.H.; Glasgow, K.C.; Thorp, H.H. *J. Am. Chem. Soc.* **1995**, 117, 8933-8938.
[2] Johnston, D.H.; Thorp, H.H. *J. Phys. Chem.* **1996**, 100, 13837-13843.
[3] For a review on quantitative RT-PCR see: Freeman, W.M.; Walker, S.J.; Vrana, K.E. *Biotechniques*, **1999**, 26, 112-125.
[4] Mertz, L.M.; Rashtchian, A. *Focus* Vol. 16, No. 2, 45-48.

Appendices

Abstract from the National M.D./Ph.D. Conference in Aspen, Colorado, July 16-18 1999.

Electrochemical Detection of Femtomole Quantities of Surface Immobilized DNA
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The oxidation of the guanine in DNA and RNA has been proposed as a possible method for the detection and quantification of clinically important nucleic acid sequences. Because of the slow electron transfer kinetics between guanine and most electrode materials an oxidation catalyst, $Ru(bpy)_3Cl_2$ (bpy = 2,2' bipyridyl), has been used to facilitate transfer of electrons from guanine to the electrode. This catalytic electrochemistry has been employed in the detection of several RT-PCR products that have been immobilized on a tin doped indium oxide (ITO) electrode.

HER-2 RT-PCR products were immobilized onto ITO electrodes. The electrodes were immersed in a solution containing 10 μM $Ru(bpy)_3$ and Cyclic Voltammetry was performed from 0-1300 mV at a scan rate of 10 V/s. Experiments involving radiolabelled RT-PCR products revealed that this catalytic system could detect less than 1 fmol (1 fmol = $1x10^{-15}$ mol) of PCR product on a 10 mm^2 electrode and could discriminate a 50 fold difference in DNA quantity. The stability of the immobilized DNA on ITO has led us to investigate whether this system could be used as a hybridization sensor to detect either single stranded cDNA or mRNA. Early experiments indicate that hybridization can be achieved; however, problems concerning non-specific attachment of the target to the electrode and poor sequence recognition need to be overcome.

For catalytic electrochemistry to become a viable technique for the detection of nucleic acid sequences the sensitivity of the system needs to improve. A system that used microelectrodes instead of the macroelectrodes used here should increase the technique's sensitivity by three orders of magnitude. At this level of sensitivity, quantification of clinically significant mRNA's, such as HER-2, could be accomplished from small tissue samples without the need for amplification.